Chapter 3

# Mycotoxin Contamination of Agricultural Products in the Southern United States and Approaches to Reducing it from Pre-harvest to Final Food Products

**Hamed K. Abbas[1] and W. T. Shier[2]**

**[1]Crop Genetics & Production Research Unit, Agricultural Research Service, United States Department of Agriculture, Stoneville, MS 38776; and [2]Dept. of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, USA**

**E-mail:** Hamed.Abbas@ars.usda.gov

Mycotoxins represent >300 fungal natural products, including aflatoxins, trichothecenes, zearalenones, ochratoxins, patulin and fumonisins. Mycotoxins contaminate cereal grains, causing acute and chronic illnesses in livestock and humans, including teratogenesis, carcinogenesis, endocrine disruption and immunosuppression. Mycotoxin-producing fungi infect plants from soil reservoirs or as endophytes, which infect seeds and plants they growing from them. Mycotoxins can be produced pre-harvest or post-harvest, but near-ideal crop handling and storage conditions in developed countries allow regulatory agencies to assume zero post-harvest production. Cereal grains range in mycotoxin contamination susceptibility from corn, the most susceptible, to rice, the least susceptible. In this paper we provide an overview of mycotoxin contamination problems in the southern United States, and give examples of efforts to reduce mycotoxin contamination pre-harvest, post-harvest and during food processing.

## Introduction

Mycotoxins are chemically-stable, toxic secondary metabolites produced by a more than 100 diverse species of fungi. More than 300 mycotoxins are now readily identified due to advances in a host of technologies including fermentation, detection, biotechnology, molecular genetics and others (*1-3*). Among the most important toxigenic fungi for agriculture and food are species of *Aspergillus*, *Fusarium* and *Penicillium*. Many other phytopathogenic fungi (e.g., *Alternaria* spp.) produce mycotoxins which are stable, toxic in experimental systems, and believed to act as virulence factors for the producing fungi. However, these mycotoxins have not been identified as food safety hazards, possibly because they do not accumulate to toxic levels in the harvested agricultural product. Some fungal species produce a single mycotoxin, while most produce multiple toxins that may or may not be chemically related. Only a handful of the 300 mycotoxins are of concern to food safety (*1,3*). These mycotoxins include aflatoxins, trichothecenes, fumonisins, ochratoxins, and zearalenone (Figure 1). Ingestion of these mycotoxins by animals and/or humans may result in toxicological problems, including teratogenic, carcinogenic, estrogenic or immunosuppressive effects (*3*). These toxicological problems can ultimately result in economic losses in agriculture (*3*, *4-12*). Because of the risks these mycotoxins pose, they have been subjected to government regulation in about 100 countries (*7*, *13*, *14*).

In principle, mycotoxins can be produced both pre-harvest and post-harvest, but in developed countries rapid drying of crops to <20% moisture (*15*) immediately after harvest, and subsequent near-ideal crop handling and storage conditions, which maintain low moisture levels, allows regulatory agencies to assume zero post-harvest production (*14*). In contrast, food and feed in developing countries are frequently contaminated with mycotoxins, which is often due to the usage of diseased plant tissues infected with a toxigenic fungus pre-harvest (*16-22*). Major crops vary widely in susceptibility to mycotoxin contamination. Corn (maize) is widely considered to be among the most susceptible of major crops to mycotoxins, while rice is among the least susceptible crops (*18-20*, *23-28*). Mycotoxins are very persistent in food and once they are present, there are no general mechanisms that will completely remove them from any type of food. Consequently, there is a need for research on improved methods to mitigate the mycotoxin problem in three areas: (i) pre-harvest to minimize mycotoxin formation in grain through effective crop management and environmental manipulation; (ii) post-harvest to prevent formation of additional mycotoxin in storage by rapid and effective drying, and to develop effective remediation methods to reduce mycotoxin levels; and (iii) during certain types of food processing to destroy mycotoxins in contaminated foods meant for direct human consumption (*29-31*). In this paper, we provide an overview of mycotoxin contamination in the crops of the southern United States; and present examples of efforts to reduce mycotoxin contamination of agricultural products by preventing pre-harvest production; by minimizing post-harvest production and by degradation during food processing.

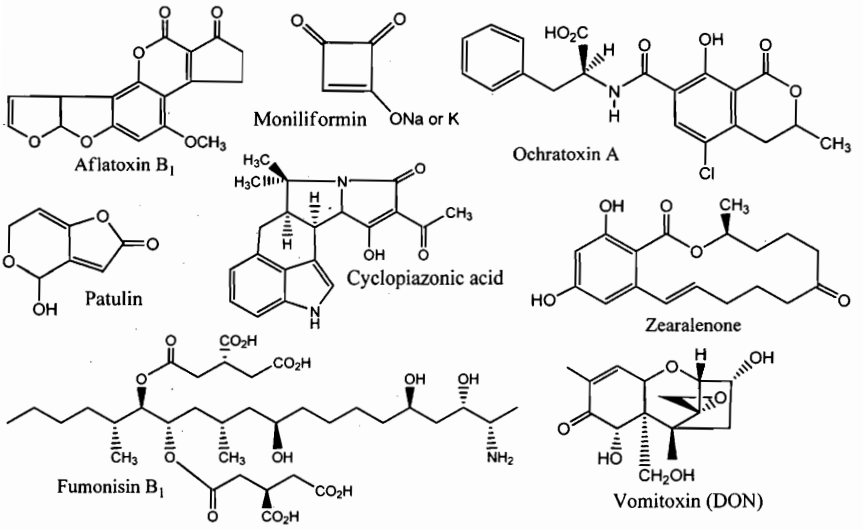


*Figure 1. Structures of some mycotoxins of interest to agriculture and food safety.*

## Mycotoxin Contamination of Corn Grown in the Southern USA

Corn (*Zea mays*) is frequently infested with fungi which produce mycotoxins and thus affect the quality and safety of food and animal feeds. Two of the most costly contamination problems are due to *Aspergillus flavus* and *Fusarium verticillioides* (syn. *F. moniliforme*) which produce aflatoxins and fumonisins, respectively (*20-22*). In 1998, both aflatoxin and fumonisin were responsible for major mycotoxin contamination problems in corn in the southern USA. The increase in mycotoxin production presumably is related to the observed weather conditions, particularly drought and high temperatures (*20, 29, 32-41*). In 1998 and 1999 corn hybrids in Mississippi and Arkansas were grown under typical agricultural practices used for the region which allowed them to be naturally infected with *Aspergillus* species and *Fusarium* spp. At harvest, corn kernel samples were evaluated for the presence of aflatoxins and fumonisins.

In 1998 all hybrids exceeded regulatory action levels of 20 ppb aflatoxin (102 - 8,100 ppb) and 5 ppm fumonisin (6 - 47 ppm). In 1999 weather conditions more closely approached 30-year norms and toxin levels were much lower than in 1998. The observed aflatoxin levels were 0 - 30 ppb and fumonisin levels were 0 - 5.8 ppm; deoxynivalenol was also detected in some samples, but no zearalenone was detected. This study supported the widely held belief that production of high levels of mycotoxins in crops by *A. flavus* and *Fusarium* spp. is associated with stress conditions, such as had occurred in 1998. The 1998 toxin levels were high enough to be of concern for food safety. Therefore, we initiated a research program focused on monitoring the

production of the mycotoxins produced in harvested corn by *Aspergillus flavus* and *Fusarium verticillioides* (Figure 2).

Although the last major aflatoxin epidemic observed in US corn was in 1998 (*20*, *21*), aflatoxin has been detected throughout our corn kernel survey, and concentrations have varied by year, location, and climatic conditions in which the samples were collected (Table 1). Similarly, in the nine years since 1998, we detected fumonisins in almost all corn samples that were analyzed (>90%) regardless of the source of the sample (Table 2). It is more common during cool, wet weather for corn to become contaminated with *Fusarium* species, notably *F. graminearum*. In cooler climates such as Minnesota, this contamination can result in a wide range of trichothecenes (particularly DON) and zearalenone (*16*, *17*). Due to the hotter, dry climate these fungi do not commonly cause disease in corn in the southern US. However, corn residues left on soil surfaces after harvest, which then over-winter in the field, contained readily-detected levels of zearalenone (0.08 to 0.80 ppm), but no deoxynivalenol (DON) (*42*).

Additional fungal species can contribute to drastically increased mycotoxin levels. In 2006 a high degree of common smut caused by *Ustilago maydis* was observed in southern US corn with ~17% of ears being infected. There was a lower incidence in 2007 (~3% infected ears), and expression of the Bt gene in the corn had no significant effect. In 2006, grain from smut-infected corn contained an average of 2437 ppm total aflatoxin, compared to ~50 ppm total aflatoxin in corn not infected with smut. In 2007, both Bt and non-Bt corn that were infected with smut had higher levels of aflatoxin and fumonisin compared to smut-free corn. Fumonisin levels were five to ten-fold higher in smut-infected corn during both years with no significant effect of the Bt status of the corn hybrids (*43*). Additional research is needed to determine how the occurrence of smut leads to increased mycotoxin contamination.

**Table 1. Aflatoxin levels in corn samples by year (µg/kg)***

| Source | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 |
|---|---|---|---|---|---|---|---|---|---|---|
| Arkansas | | | | | | | | | | |
| Number | 298 | 120 | nd | 121 | 161 | 122 | 69 | 64 | nd | nd |
| Range | 5-4544 | 5-114 | nd | 6-525 | 7-224 | 5-1390 | 11-550 | 5-122 | nd | nd |
| % Positive | 91 | 30 | nd | 5 | 2 | 31 | 14 | 20 | nd | nd |
| Georgia | | | | | | | | | | |
| Number | nd | nd | nd | nd | nd | nd | nd | nd | 646 | nd |
| Range | nd | nd | nd | nd | nd | nd | nd | nd | 5-572 | nd |
| % Positive | nd | nd | nd | nd | nd | nd | nd | nd | 13 | nd |
| Illinois | | | | | | | | | | |
| Number | nd | nd | nd | nd | nd | nd | nd | nd | 83 | 87 |
| Range | nd | nd | nd | nd | nd | nd | nd | nd | 20-1385 | 5-1425 |
| % Positive | nd | nd | nd | nd | nd | nd | nd | nd | 100 | 99 |
| Louisiana | | | | | | | | | | |
| Number | nd | nd | nd | 552 | 843 | 882 | 211 | 95 | 96 | 176 |
| Range | nd | nd | nd | 5-34950 | 5-10600 | 5-3200 | 5-2305 | 5-2435 | 6-55 | 17-1470 |
| % Positive | nd | nd | nd | 63 | 74 | 56 | 32 | 86 | 8 | 83 |
| Mississippi | | | | | | | | | | |
| Number | 25 | 103 | 489 | 506 | 249 | 104 | 92 | 46 | 46 | 450 |
| Range | 41-590 | 5-30 | 5-2665 | 6-605 | 5-3120 | 5-195 | 5-427 | 7-143 | 5-1710 | 14-175 |
| % Positive | 72 | 32 | 52 | 12 | 53 | 12 | 55 | 37 | 65 | 2 |
| Tennessee | | | | | | | | | | |
| Number | nd | nd | nd | nd | nd | 72 | nd | nd | nd | nd |
| Range | nd | nd | nd | nd | nd | 5-7 | nd | nd | nd | nd |
| % Positive | nd | nd | nd | nd | nd | 3 | nd | nd | nd | nd |
| Texas | | | | | | | | | | |
| Number | nd | nd | nd | nd | nd | nd | 80 | nd | nd | nd |
| Range | nd | nd | nd | nd | nd | nd | 10-1692 | nd | nd | nd |
| % Positive | nd | nd | nd | nd | nd | nd | 95 | nd | nd | nd |

Abbreviation: nd = not done

* Aflatoxin levels were measured by commercial ELISA assays with limit of detection = 5 µg/kg. Data for Mississippi in 1998 was supplied by Dr. Gary Windham, Starksville, MS.

**Table 2. Fumonisin levels in corn samples by year (µg/g)***

| Source | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 |
|---|---|---|---|---|---|---|---|---|---|---|
| Arkansas | | | | | | | | | | |
| Number | 256 | 116 | nd | 120 | 161 | 122 | 69 | 72 | nd | nd |
| Range | 4-128 | 0.5-12 | nd | 4-170 | 0.5-58 | 0.5-17 | 0.6-52 | 0.6-23 | nd | nd |
| % Positive | 100 | 78 | nd | 100 | 81 | 84 | 99 | 88 | nd | nd |
| Georgia | | | | | | | | | | |
| Number | nd | nd | nd | nd | nd | nd | nd | nd | 168 | 673 |
| Range | nd | nd | nd | nd | nd | nd | nd | nd | 0.5-24 | 0.5-155 |
| % Positive | nd | nd | nd | nd | nd | nd | nd | nd | 43 | 98 |
| Illinois | | | | | | | | | | |
| Number | nd | nd | nd | nd | nd | nd | nd | nd | 83 | 88 |
| Range | nd | nd | nd | nd | nd | nd | nd | nd | 0.5-13 | 0.5-42 |
| % Positive | nd | nd | nd | nd | nd | nd | nd | nd | 73 | 78 |
| Louisiana | | | | | | | | | | |
| Number | nd | nd | 706 | 550 | 1,676 | 1,728 | 935 | 969 | 595 | 176 |
| Range | nd | nd | 0.5-32 | 0.5-14 | 0.5-68 | 0.5-460 | 0.5-176 | 0.5-51 | 0.5-20 | 0.5-18 |
| % Positive | nd | nd | 93 | 77 | 95 | 95 | 92 | 87 | 60 | 78 |
| Mississippi | | | | | | | | | | |
| Number | 312 | 511 | 838 | 599 | 790 | 887 | 714 | 313 | 384 | 683 |
| Range | 2-74 | 0.5-13 | 0.5-32 | 0.5-50 | 0.5-43 | 0.5-50 | 0.5-60 | 0.5-64 | 0.5-28 | 0.5-32 |
| % Positive | 100 | 89 | 82 | 98 | 95 | 96 | 89 | 90 | 82 | 39 |
| North Carolina | | | | | | | | | | |
| Number | nd | nd | nd | nd | nd | nd | nd | nd | 120 | nd |
| Range | nd | nd | nd | nd | nd | nd | nd | nd | 0.5-11 | nd |
| % Positive | nd | nd | nd | nd | nd | nd | nd | nd | 67 | nd |
| Tennessee | | | | | | | | | | |
| Number | nd | nd | nd | nd | nd | 72 | nd | nd | nd | nd |
| Range | nd | nd | nd | nd | nd | 0.5-36 | nd | nd | nd | nd |
| % Positive | nd | nd | nd | nd | nd | 99 | nd | nd | nd | nd |
| Texas | | | | | | | | | | |
| Number | nd | nd | nd | nd | nd | 18 | 80 | nd | nd | nd |
| Range | nd | nd | nd | nd | nd | 4-24 | 0.8-69 | nd | nd | nd |
| % Positive | nd | nd | nd | nd | nd | 100 | 98 | nd | nd | nd |

Abbreviation: nd = not done

* Fumonisin levels were measured by commercial ELISA assays with limit of detection = 0.5 µg/g.

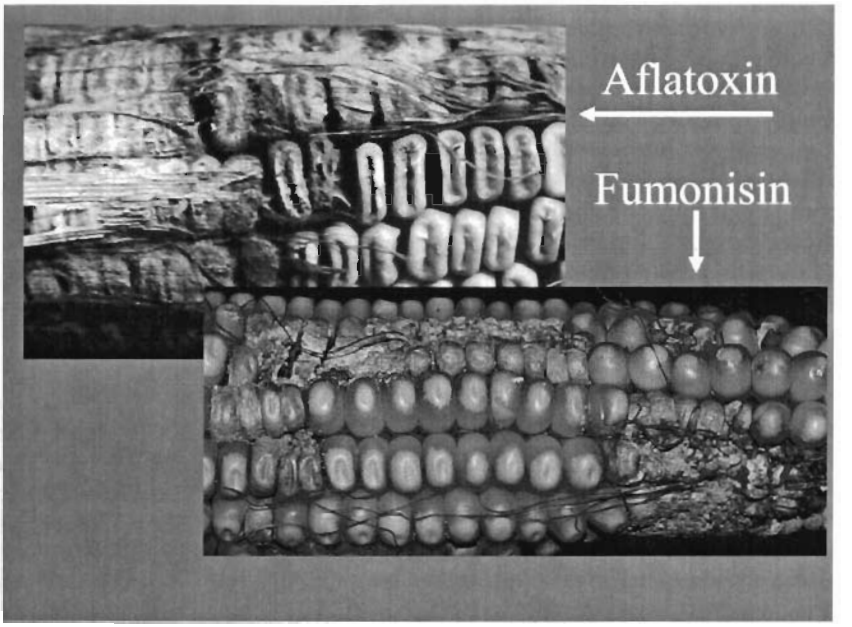


*Figure 2. Corn (maize) cobs infected with fungi that produce the mycotoxins aflatoxin (Aspergillus flavus) and fumonisin (Fusarium verticillioides) (syn. F. moniliforme).*

## Mycotoxin Contamination of Rice Grown in the Southern USA

Rice is widely regarded as unusually resistant to mycotoxin contamination, despite having kernels that provide an exceptionally favorable culture medium for fungal growth and mycotoxin production. We are interested in learning why rice is mycotoxin-resistant, and if any of the resistance mechanisms can be transferred to mycotoxin-susceptible crops such as corn. Among the plausible mechanisms for mycotoxin resistance in rice are the following: (i) rice kernels develop and mature in a structure anatomically resistant to fungal infection, due at least in part to the kernels being physically separated so a fungal infection can not spread directly from kernel-to-kernel, as it can in corn; and (ii) all upland rice and most paddy rice is harvested in the dry season in Asia, which ensures that the crop is effectively dried and that it spends most of its storage time under near ideal conditions. In contrast, most corn is grown in regions where there is no reliable cycle of wet and dry seasons to synchronize with.

In order to gain information about plausible explanations for the resistance of rice to mycotoxin contamination, we have examined rice harvested in situations in which one of the proposed resistance mechanisms was not operative. To investigate the importance of the first mycotoxin resistance mechanism proposed above (i.e., mycotoxin resistance is due to the resistance of rice kernels to fungal infection), we examined rough rice samples (67 in Arkansas in 1998; 33 in Mississippi in 1999 and 2000) harvested from fields

exhibiting *Fusarium* sheath rot disease, in which *F. proliferatum* had succeeded in infecting lower parts of the rice plants. Of the samples tested, 27% contained *F. proliferatum* (84% toxigenic); 11% contained fumonisins (0.5-6.2 ppm). A sample of rough rice with 5.13 ppm fumonisin yielded hulls with 16.8 ppm fumonisin; brown rice with 0.87 ppm; and bran with 3.53 ppm; but no fumonisin was detected in polished rice (*19*, *37*). To examine the importance of the second mycotoxin resistance mechanism proposed above (i.e., rice is stored under near ideal conditions), we measured mycotoxin levels in rice samples that had gotten wet during storage. No fumonisin was detected, but aflatoxin was present in all samples of rough rice studied (average, 143 ppb; range: 50-272 ppb). A sample of rough rice with 61.8 ppb aflatoxin yielded hulls with 16.3 ppb aflatoxin; brown rice with 88.3 ppb; bran with 587 ppb; polished rice with 63.5, and it yielded cooked rice with 28.4 ppb aflatoxin. Thus, the results obtained so far are consistent with mycotoxin resistance in rice being due at least in part to resistance to fungal infection and near ideal processing and storage conditions. However, the observations of mycotoxins in rice hulls and bran at much higher levels than in the endosperm, the part people consume, is consistent with the presence of an unidentified barrier(s) to endosperm contamination, which would represent an additional protective mechanism different from the separation of kernels mechanism discussed above.

Rice samples which showed symptoms of *Fusarium* sheath rot were collected during the 1995 harvest season from Arkansas (7 samples) and Texas (13 samples). Fumonisins ($FB_1$ and $FB_2$) were isolated from 40% of the rice samples tested that showed symptoms of *Fusarium* sheath rot. The level of fumonisins was $\leq$4.3 ppm. *Fusarium proliferatum* isolates were obtained from these rice samples and shown to produce fumonisins at levels of 14 to 230 ppm. Also, these isolates produced moniliformin in a range of 7 to 6018 ppm and beauvericin at 109 to 1350 ppm. Shelling and milling of unpolished rice showed that fumonisin levels were very high in hulls ($\geq$17 ppm), low in brown rice ($\leq$0.9 ppm), moderate in bran ($\leq$4 ppm), but were below the level of detection in polished rice.

More than 100 rice samples were selected out of thousands collected by a farmer's cooperative in the Mid-South area during an outbreak of scab disease caused by *Fusarium graminearum*. These samples were analyzed for scab disease, *Fusarium* spp., deoxynivalenol (DON, vomitoxin), and zearalenone. DON was detected in 94 of 100 samples at 0.1 to 1.6 ppm (ELISA) when naturally-contaminated rice kernels were extracted with water. DON and zearalenone levels measured by ELISA were affected by the type of extraction solvent used. DON was detected by GC/MS in 38% of samples (0.1 to 0.4 ppm) and zearalenone was detected by GC/MS in 65% of samples (0.3 to 2.2 ppm). Samples were analyzed for aflatoxin and fumonisins by ELISA as well. Aflatoxins were found in 10 out of 100 samples at levels of 20.2 to 28.6 ppb and fumonisins were found in 5 out of 80 samples at levels of 0.1 to 0.5 ppm. Representative isolates of *F. graminearum* produced DON and its derivatives (7 to 430 ppm) and zearalenone (583 to 9,883 ppm) (*45*).

## Mycotoxin Stability in Stored Agricultural Products

There has been extensive research on remediation methods to reduce mycotoxin contamination in stored grain for use in animal feeds, or at least to reduce the toxicity. Mycotoxin stability in agricultural products depends heavily on the chemical and physical properties of the mycotoxins. Aflatoxins in commodity products are considered very stable compounds based on their physical properties. Aflatoxins are highly reactive with alkaline oxidizing agents, such as sodium hypochlorite. Therefore these agents are usually used in many laboratories to inactivate aflatoxin on glassware and laboratory bench surfaces. Most research on removal of aflatoxin from agricultural products has focused on "ammoniation" (treatment of dry grains or milled products with anhydrous ammonia) (*46-48*), screening out fines (fungus-infected kernels are more likely to break than sound ones, so removing broken kernels reduces the aflatoxin level) and incorporation of additives such as montmorillite clay or charcoal to bind toxin in the gastrointestinal tract (*49*). Aflatoxin can be degraded by urea and sunlight (*50, 51*). Aflatoxins in foods can be reduced by cooking and extruding (*33, 34*), techniques in which relatively high temperatures and pressures were applied. Roasting temperatures ranging from 143 to 149°C reduced aflatoxin concentrations in corn to about 50% of original values (*52*). Fumonisins were found to be reduced by 50% to other detectable fumonisins derivatives such as hydrolyzed fumonisin analogs in tortillas using the traditional nixtamalization method (*53, 54*). Recently, Voss et al. (*55*) reported that extrusion processing of corn grits in the presence of a reducing sugar (*56*), glucose, significantly reduced the toxicity of contaminating fumonisin in rats. Cramer and Humpf reported that ochratoxin A can be reduced by 30% to 80% depending on temperatures used in dry heating or during roasting processes. They found that ochratoxin A degradation products were formed, which were less toxic than the parent compound (*57*). Patulin can be removed from apple and apple products by using the correct temperature for storage before processing and also by removing damaged portions of the fruit before processing (*58*).

Aflatoxins are sufficiently stable in food and food products that the heat in cooking, baking, frying, and autoclaving (Table 3), and the enzymes in brewing reduced but did not destroy all the aflatoxin (Abbas, unpublished results). We have found that shelling, milling, baking, and cooking did not remove aflatoxin, DON, zaralenone, and fumonisin from rice, wheat, or corn products (*59*). These results showed that aflatoxin persists in rice even when it is boiled and cooked for about 40 minutes (*59*), whereas cooked rice became free from extractable fumonisin (*18*).

It is widely assumed that the mycotoxin content of agricultural products remains constant as long as they are stored under relatively dry conditions (i.e., less than 15% moisture content). Thus, the expense of determining mycotoxin content at one point post-harvest need not be repeated. We have tested this assumption by tracking the aflatoxin (Figure 3) and fumonisin (Figure 4) contents of naturally-contaminated corn over a period of one year under various storage conditions. We observed that the levels of aflatoxins and to a lesser extent, levels of fumonisins dropped significantly during the last six months of

storage in the dark at both 35°C and -20°C. The reduction in mycotoxin levels occurred whether or not the corn meal was autoclaved first to inactivate degradation enzymes from the corn or any microbial contaminants. The destruction of the mycotoxins in the dark without effects of enzymes or storage temperature is most consistent with destruction of the mycotoxins by an oxidative or peroxidative process(es) following the depletion of endogenous antioxidants.

**Table 3. Effect of heat treatment on aflatoxin contamination levels in harvested corn kernels.**

| | Aflatoxin levels (ppb) | | | | % reduction of |
|---|---|---|---|---|---|
| | Not autoclaved | | Autoclaved* | | total aflatoxin |
| Corn sample | AFB1 | AFB2 | AFB1 | AFB2 | by heat |
| Sample 1 | 414 | 7.4 | 172 | 3.8 | 42 |
| Sample 2 | 578 | 18 | 165 | 4.5 | 29 |

* Autoclaved = heated at 120°C for 15 minutes

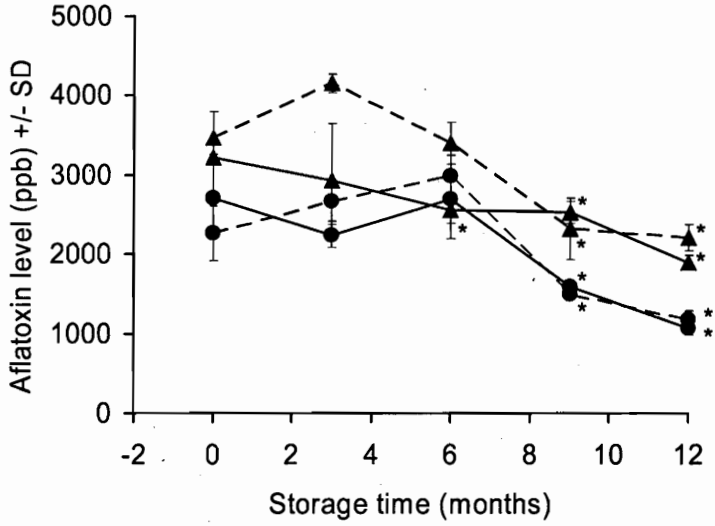


*Figure 3. The effect of storage time on aflatoxin levels in ground corn which was either autoclaved at 120°C for 15 minutes to inactivate enzymes in the corn and any microbial contaminants which might degrade the toxin (solid triangles), or left unheated (solid circles). Triplicate samples were stored in either a warm environment, 35°C (solid lines), or in a freezer at -20°C (dashed lines). Aflatoxin levels were measured with commercial ELISA assays. (* = significantly less than at zero months, Student's t-test, $P<0.05$; SD = standard deviation).*

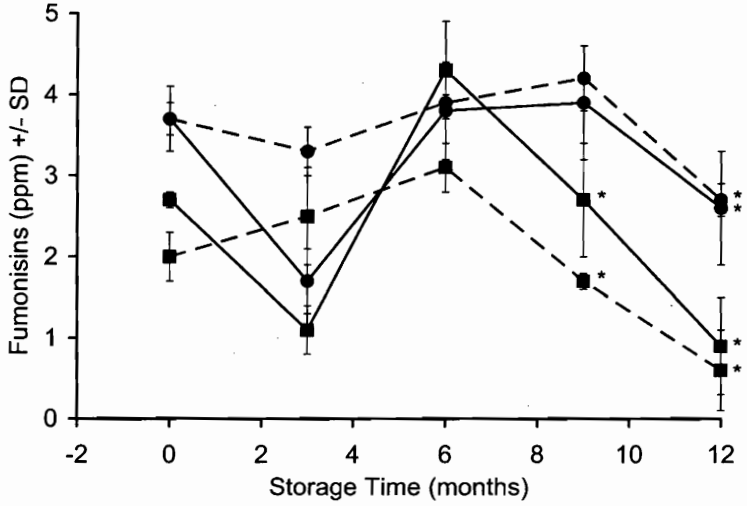


*Figure 4. The effect of storage time on fumonisin levels in ground corn which was either autoclaved at 120°C for 15 minutes to inactivate enzymes in the corn and any microbial contaminants which might degrade the toxin (solid circles), or left unheated (solid squares). Triplicate samples were stored in either a warm environment, 35°C (solid lines), or in a freezer at -20°C (dashed lines). Fumonisin levels were measured with commercial ELISA assays. (* = significantly less than at six months, Student's t-test, $P<0.05$; SD = standard deviation).*

## Effect of Food Processing on Mycotoxin Content

Government regulations in developed countries require foods intended for direct human consumption to be made from grains with low levels of mycotoxin contamination (e.g., aflatoxins less than 20 ppb in the United States and less than 5 ppb in Europe), even when some standard food processing techniques are known to reduce mycotoxin contamination levels. Thus, the effects of food processing-induced changes in the structures or content of regulated mycotoxins is generally not a concern in the US and other developed countries, as long as food processing doesn't convert mycotoxins to forms with greater toxicity or higher bioavailability. Changes in the structures of biologically active compounds usually result in destruction or reduction of activity. This rule of thumb appears to apply to most mycotoxins as well, but several lines of research from numerous laboratories indicate that fumonisins are an exception to this rule.

## Food Processing-Induced Changes in Fumonisins

The first indication that fumonisins might be unusual in their ability to retain activity despite molecular modification was the observation (*60*) that hydrolyzing the side chains off of fumonisin $B_1$ gave hydrolyzed fumonisin $B_1$ ($HFB_1$) which retained substantial cytotoxic activity with cultured mammalian cells. Intact fumonisin $B_1$ ($FB_1$) exhibited strong cytotoxic activity with some cell lines that exhibit significant levels of differentiated properties in culture, including epithelial cell lines (e.g., MDCK, PK-15, Caco-2) and liver cell lines (e.g., H4TG), but the more commonly used undifferentiated cell lines were resistant. In contrast, $HFB_1$ exhibited a lower level of cytotoxic activity, but was cytotoxic with all cell lines. It has not been established whether the selective toxicity of $FB_1$ in the differentiated cell lines is related to higher levels of transport into the cells, or higher levels of metabolism, or both. $HFB_1$ has been studied by many investigators in a variety of contexts including its formation during food and feed processing, its toxicity demonstrated in some feeding studies, and its detection in commercial food products (*56*, *61-66*).

Biogenic and abiogenic changes in the structures of fumonisins during food processing can, in principle, lead to increased toxicity by various mechanisms including increased bioavailability, altered metabolism, and inherently increased toxicity by tighter binding to its site of action.

### a) Food Processing-induced Changes to Fumonisins Which May Increase Bioavailability

There are many unanswered questions about the bioavailability of fumonisins (*66*). Studies by numerous research groups on the toxicokinetics of radiolabeled and unlabeled fumonisins in numerous animal species have indicated that they are poorly absorbed after oral administration. Bolger et al. (*67*) have summarized these studies by concluding that absorption of $FB_1$ is negligible after oral administration (4% of dose). Studies have also provided evidence that fumonisins undergo very limited, if any, functional or non-functional metabolism (reviewed in *66*), except $HFB_1$ (*68*). Nevertheless, fumonisins exhibit readily demonstrated toxic effects in some species, particularly horses, but toxicity has been more difficult to demonstrate in other species, including humans for whom the evidence is limited to correlations between exposures and higher rates of cancer and neural tube birth defects in certain areas. The existence of robust toxic effects of oral $FB_1$ in some species, but very low oral absorption has been called the "fumonisin paradox" (*66*). There are various possible explanations for this apparent paradox, including the following: (i) fumonisins act by a cascading mechanism of action, which amplifies the small response to the 4% which is absorbed, into readily measured toxicity; (ii) fumonisins exhibit greatly increased bioavailability/metabolism at environmental concentrations, which are generally much lower than those used in the reported toxicokinetic studies; or (iii) abiogenic conversion of fumonisins occurs during processing of foods and feeds, which converts fumonisins to a form(s) with much greater bioavailability than the unaltered fumonisins used in

the reported toxicokinetic studies. Abiogenic conversion products would have to either retain biological activity or be efficiently converted back to active fumonisin forms once inside the body.

The "fumonisin paradox" has yet to be resolved, but experiments carried out in our laboratories (*69*, *70*) and others (*71*, *72*) have provided additional information about the possible species that can be formed abiogenically during normal food processing and might provide a resolution similar to option (iii) given above. $FB_1$ was biosynthetically radiolabeled by feeding *Fusarium verticillioides* cultures methionine radiolabeled with tritium on the S-methyl group. Tritium in this position of methionine becomes incorporated into the $FB_1$ backbone on methyl side chains. A tritium label at this point in the fumonisin molecule is as chemically stable as a carbon-14 label would be. Incorporating radiolabeled $FB_1$ into corn meal and roasting it in a laboratory model of corn flake manufacturing resulted in greater than 90% of the radiolabel being covalently bound to a water-insoluble, detergent solution-soluble, non-dialyzable, trichloroacetic acid-precipitable substance, consistent with the toxin being covalently bound to protein. As a result the radiolabeled $FB_1$ was in a form not extractable by the aqueous methanol or acetonitrile solvent systems used to determine the $FB_1$ content of processed foods and feeds. Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis with autoradiographic detection indicated most of the label was bound to non-zein proteins, with very little bound to zeins, the major storage proteins in corn kernels. This observation is consistent with radiolabeled $FB_1$ binding predominantly to the ε-amino groups of lysine residues in protein, which are absent from zeins. Alkaline hydrolysis released the radiolabel from protein to give intact radiolabeled $HFB_1$ in high yield, consistent with radiolabeled $FB_1$ binding to protein through one of its side chains. Heating solid $FB_1$ alone at temperatures encountered in roasting yielded non-dialyzable, presumably polymeric material that gave intact $HFB_1$ in high yield on alkaline hydrolysis. $FA_1$, which has the free amino group of $FB_1$ blocked with an acetyl group, heated alone at temperatures encountered in roasting yielded mixtures containing a substance with NMR and mass spectral properties consistent with N-acetyl-$FB_1$-anhydride, but it was too unstable for isolation and rigorous structural confirmation. These results are consistent with one of the tricarballyl side chains of $FB_1$ loosing a molecule of water to generate an succinic anhydride moiety, which can then react with whatever free amino group is most abundant, which are ε-amino groups of lysine residues in non-zein proteins in food products derived from corn (see Figure 5). In the course of the reaction the ring of the cyclic anhydride side chain is opened and the $FB_1$ becomes covalently bound to protein through an amide linkage.

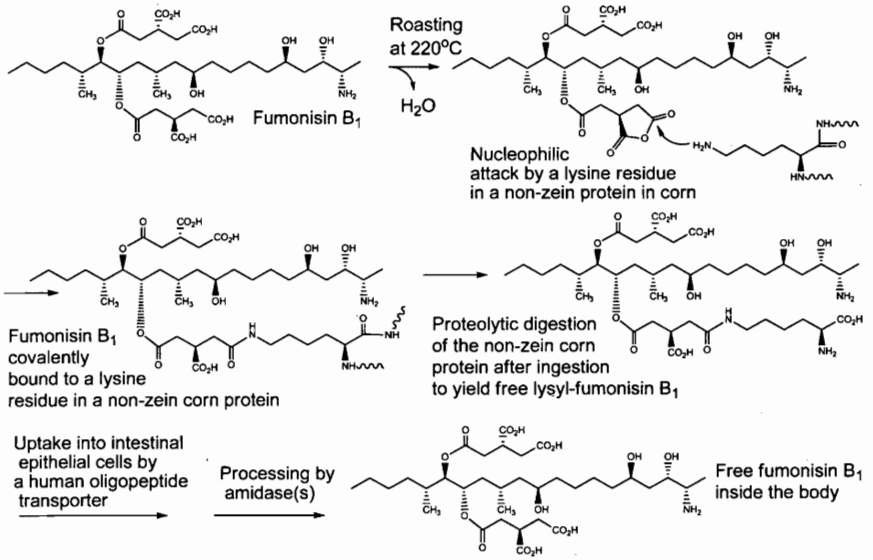


*Figure 5. A proposed mechanism for covalent binding of tritium labeled fumonisin $B_1$ to e-amino groups of lysine residues in non-zein corn proteins in a laboratory model of corn flake manufacture.*

When first discovered (*63*), the disappearance of extractable, HPLC-detectable $FB_1$ from corn-derived foods during heating involved in food processing was encouraging, and taken as an indicator that toxicity also disappeared. However, in subsequent research Scott and associates (*71*, *72*) showed that the amount of $HFB_1$ released from corn flakes, tortilla chips and corn chips by alkaline hydrolysis corresponded to about twice as much $FB_1$ being present in bound form, presumably through a side chain, as was present in free form. Identifying the reaction that forms a side chain anhydride with subsequent covalent coupling to the ε-amino groups of lysines in proteins provides a plausible mechanism for the binding of $FB_1$ to corn components. However, it is also a source of concern, because attachment to an amino acid provides a mechanism for efficient uptake of bound $FB_1$ from the gastrointestinal tract. There exist various peptide transporters in intestinal epithelial cells, the best characterized of which is oligopeptide-T, which has been shown to be relatively promiscuous in its transporting specificity (*73*). Not only does oligopeptide-T transport into the body miscellaneous di- and tri-peptides generated by the partial digestion of proteins, it also transports various drugs, including valcyclovir and penicillins (for examples, see Figure 6), giving them much higher bioavailability than would otherwise be expected. The oligopeptide transporter appears to transport particularly well drugs that are about the size of a di- or tri-peptide with an amino acid attached to a moiety with a wide range of possible structures. Lysyl-$FB_1$ fits within the range of structures which might be transported by oligopeptide-T and some other transporters, but the transport characteristics of lysyl-$FB_1$ have not yet been studied.

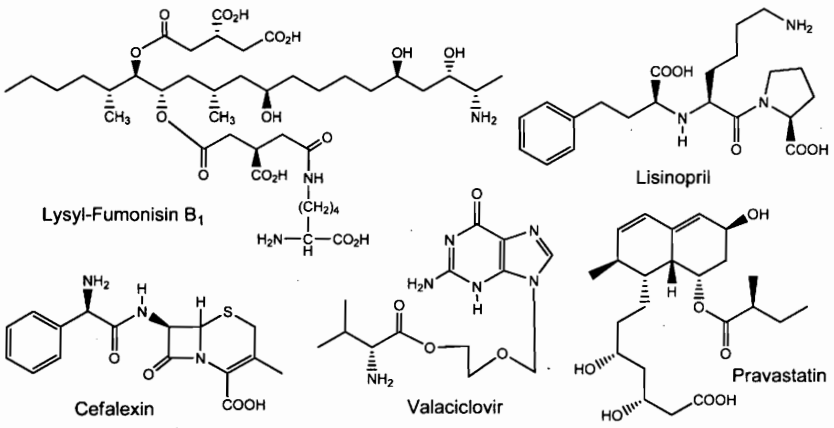


*Figure 6. Comparison of the structure of lysyl-fumonisin $B_1$ with drugs known to be transported by the human intestinal oligopeptide transporter, hPepT1, and related transporters*

**b) Food Processing-induced Changes to Fumonisins Which May Increase Toxicity**

As indicated above, removing the side chains from $FB_1$ to give $HFB_1$ reduces, but does not completely destroy biological activity. Side chain hydrolysis has also served to reduce concern about fumonisin toxicity in processed foods derived from corn, because the majority of corn consumed directly by humans is subjected to lime (calcium hydroxide) water treatment in a process known as nixtamalization. Nixtamalization is used to remove the pericarp from corn kernels and soften the rest of the kernel, making it easy to grind into masa, the form of corn used to make tortillas, the major starchy staple in the Mexican diet. Nixtamalization has the additional advantage that some of the fumonisins and hydrolyzed fumonisins are extracted into the lime water, which is discarded. Masa is also used to make tortilla chips and corn chips in the US.

In 1998 Humpf et al. (*74*) discovered that N-palmitoyl-$HFB_1$ not only retains *in vitro* toxicity, but exhibits about ten-fold greater toxicity than the original intact $FB_1$. This observation was confirmed (*70*) in an investigation of the structure-activity relationships for *in vitro* toxicity of N-fatty-acyl derivatives of intact and hydrolyzed fumonisins. These studies showed that optimal cytotoxicity occurred with N-fatty acyl-$HFB_1$ derivatives with carbon lengths of 8 to 12 (see Figure 7). Longer chain lengths (C-14 and C-16) of N-fatty acyl-$HFB_1$ derivatives retained most of the toxicity observed at the optimum, whereas shorter chain lengths (down to acetate, C-2) were much less active. Polyunsaturation in the fatty acid moieties of N-fatty acyl-$HFB_1$ derivatives resulted in slight reductions in cytotoxic activity. In contrast, all N-fatty acyl derivatives of intact fumonisins exhibited no cytotoxic activity. The

observations of *in vitro* toxicity of N-fatty acyl-$HFB_1$ derivatives combined with the results of experiments carried out in our laboratories on abiogenic conversions of radiolabelled $FB_1$ and radiolabelled $HFB_1$ in laboratory models of tortilla chip manufacture raise concerns about the safety of nixtamalized, fried corn products in the American diet, including taco shells, corn chips and tortilla chips.

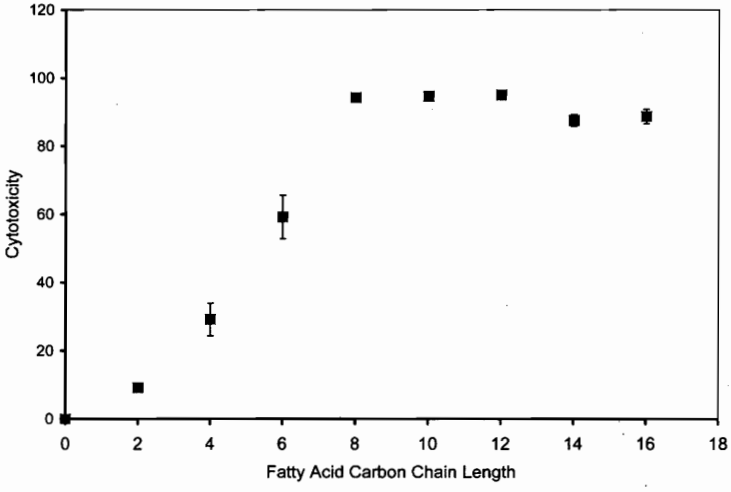


*Figure 7. The effect of fatty acid chain length on cytotoxicity of N-fatty acyl hydrolyzed fumonisin $B_1$ derivatives. Cytotoxicity is expressed as the percent reduction in the concentration of N-fatty acyl hydrolyzed fumonisin $B_1$ derivative required to cause a 50 percent reductionin cell proliferation in four cultured mammalian cell lines.*

It has been well established (*75*, *76*) that N-fatty acylation occurs during food processing, particularly frying. The reaction has been most extensively studied for acylation of the ε-amino groups of lysine in proteins, which has been investigated to determine if there is a significant effect on the nutritional value of fried foods. In principle, N-fatty acylation of $HFB_1$ in nixtamalized corn can occur by at least two mechanisms. In the simplest mechanism, acylation can occur by direct acyl transfer as a result of nucleophilic attack of free amino groups on the ester moieties of triglycerides. However, several lines of evidence suggest that the majority of N-acylation occurs with intermediate formation of fatty acid anhydrides or other reactive species by thermal decomposition of triglycerides (Figure 8). Any fatty acid anhydride formed will react rapidly with free amines, including $HFB_1$ to form N-fatty acyl-$HFB_1$ derivatives. Lime-treated corn is distinctly basic, so most amino groups are in the reactive unprotonated form. The studies in this laboratory examined the possibility that N-fatty acyl-$HFB_1$ derivatives would form from added radiolabled $HFB_1$ in a laboratory model for the frying of corn chips in pre-heated vegetable oil. Tortilla chips and corn chips are made from lime-treated corn and standard manufacturing processes always fry them in a mixture of new and pre-heated

oil. Tortilla chips contain about 30% oil absorbed from the frying bath. The oil removed in the chips is continuously replaced with new oil, so that no used cooking oil is ever discarded, and all mycotoxins extracted out of the masa during frying end up in other chips, if they are not destroyed by the heat. Corn chips differ from tortilla chips in that they are fried at higher temperatures (185-210°C for corn chips *vs* 170-190°C for tortilla chips) and they are thicker, so that they retain more oil (*77*, *78*). We observed in the laboratory model that essentially all tritium-labled $HFB_1$ underwent a chemical reaction during frying. About 90% of the radiolabelled $HFB_1$ was converted to N-fatty acyl-$HFB_1$ derivatives, which were efficiently extracted out of the chips into the frying oil. The remaining 10% of radiolabel was protein-bound in the chip or converted to polar substances extracted into the oil. Radiolabled $FB_1$ is also converted to N-fatty acyl derivatives (about 85% conversion) during frying in this laboratory model, but the extraction efficiency into the cooking oil was lower (about 80%).

Efficient conversion of $HFB_1$ to N-fatty acyl-$HFB_1$ derivatives with ten times the *in vitro* toxicity of $FB_1$ during food processing is a food safety concern that is only partially compensated for by reduction in toxicity by N-fatty acylation of intact $FB_1$. The observation that the conversion products are efficiently extracted into the cooking oil is of no practical importance with current manufacturing practices, because they will be absorbed into other chips along with the cooking oil. However, the phenomenon does present an opportunity to remove a potential carcinogen from food during processing, if efficient scrubbers could be developed to continuously remove the N-fatty acyl-$HFB_1$ derivatives from the oil during frying. However, it will first be necessary to demonstrate that N-fatty acyl-$HFB_1$ derivatives are both present in foods and toxic *in vivo*.

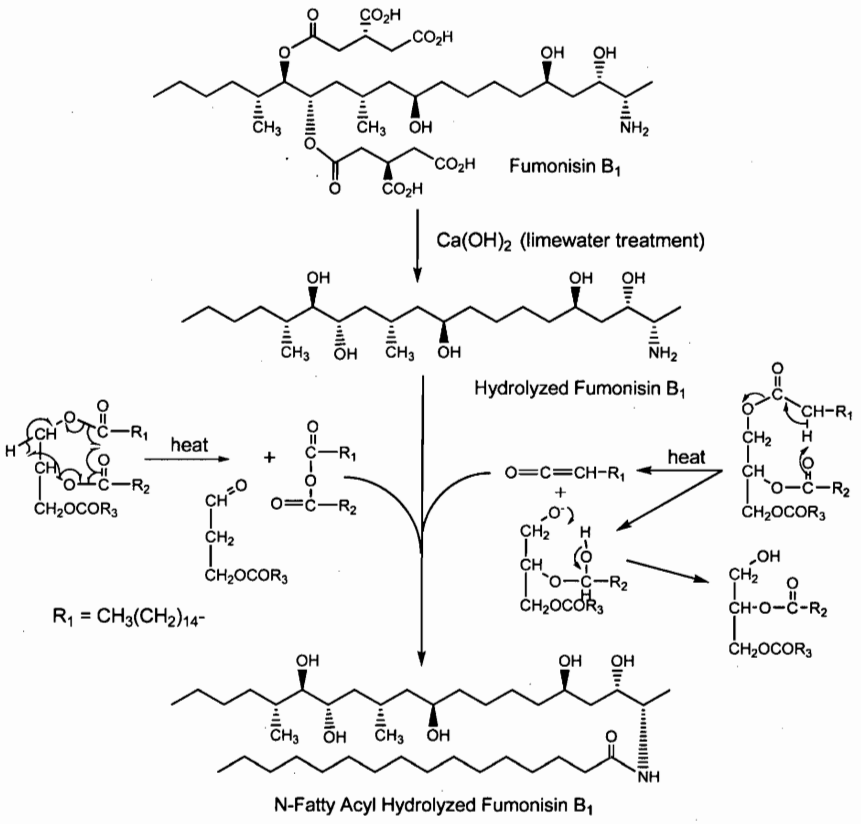


*Figure 8. Proposed mechanisms for the formation of N-fatty acyl hydrolyzed fumonisin $B_1$ derivatives from tritium labeled hydrolyzed fumonisin $B_1$ in a laboratory model of corn chip manufacturing.*

## Conclusion

While substantial progress has been made in developing methods to reduce mycotoxin contamination in foods and feeds, more research is needed to ensure that completely safe foods and feeds will continue to be available in the future.

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